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Article type : Letter to the Editor

Gut microbiota in wheezing preschool children and the association with childhood asthma.

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14156](https://doi.org/10.1111/ALL.14156)

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To the editor,

Reliable biomarkers to predict asthma in wheezing preschool children are lacking. Recently, the impact of gut microbial perturbations on the development of asthma gained widespread attention. Gut microbial dysbiosis in the first year of life was associated with asthma in multiple birth cohort studies [1-5]. Microbial metabolites might play a crucial role in maintaining an adequate immune balance and preventing asthma through its influence on regulatory T-cells (Tregs) and the *Foxp3* gene [1, 6]. However, most data are derived from animal studies, whereas most human studies have focused on the association between infant gut microbiota and asthma-like symptoms at an age when a reliable diagnosis of asthma cannot yet be made. Furthermore, no studies have been performed that investigated gut microbial composition in wheezing children, and its association with subsequent development of asthma.

In the Asthma DEtection and Monitoring (ADEM) study (clinicaltrial.gov: NCT 00422747), 202 wheezing children and 50 healthy controls aged 2 to 4 years were prospectively followed until 6 years of age, when a definitive diagnosis of asthma was made. The study was approved by the Dutch national medical ethical committee, and written informed consent was given by all parents. A detailed study protocol was previously published [7, 8].

At inclusion, faecal and blood samples were collected. Faecal microbial composition was analysed by sequencing of the 16S rRNA V3-V4 gene region. In total, 230 samples (70 true asthmatics, 114 transient wheezers, and 46 healthy controls) were successfully analysed (see flow chart, supplementary figure S1). In blood, atopic sensitisation (Phadiatop Infant test), proportion of Tregs by flow cytometry (CD4⁺CD25^{high}CD127⁻), and *Foxp3* gene expression were assessed. See supplementary material for a detailed description of study methods. The baseline characteristics are displayed in supplementary Table S1.

First, we examined whether microbial richness and diversity at preschool age were predictive for future asthma development. Neither the microbial richness (OR 0.99 [95%CI 0.98-1.01]; p = 0.46), nor the microbial diversity as assessed by the Shannon index (OR 1.01 [0.98-1.04]; p = 0.53) were significantly different between transient wheezing children and true asthmatics while adjusting for potential confounders (sex, breastfeeding, birth season, atopy parents, siblings, parental smoking status, day care attendance) (Figure 1A-B). At preschool age, these indices were also not different between wheezers and healthy controls, while adjusting for potential confounders (Figure S2).

Next, we examined the overall microbial community structure (as assessed by the Bray-Curtis dissimilarity), which was neither significantly different between transient wheezers and true asthmatics (PERMANOVA $p = 0.07$, Figure 1C), nor between preschool wheezers and healthy controls (PERMANOVA $p = 0.22$, Figure S2). Microbial profiles of all children were clustered using Dirichlet Multinomial Mixture (DMM) modelling. Three distinct clusters (enterotypes) were identified, i.e. those that were driven by a relatively high abundance of *Bifidobacterium*, *Bifidobacterium* combined with *Blautia*, and *Prevotella* combined with *Bifidobacterium* respectively. Neither the amount of wheezing children who developed asthma, nor the proportion of children with preschool wheeze were significantly different among the three enterotypes, while adjusting for multiple confounders in multivariable logistic regression analyses (Table S2 and S3). Altogether these results indicate that microbial diversity and overall microbial community structure are not predictive for subsequent asthma development among preschool wheezing children. Furthermore, we examined whether the relative abundance of specific bacterial genera was predictive for future asthma development. Using multivariable logistic regression, we found that the relative abundance of the genera *Gemmiger* ($p = 0.03$) and *Escherichia* ($p = 0.02$) was significantly higher in wheezing children who developed asthma at age 6 years (Figure 2A-B). The risk of developing asthma was highest in those children who harboured the highest relative abundance of these two bacterial genera (Figure 2C-D). In particular, a high relative abundance of *Escherichia* was associated with 4.6-fold increased odds of asthma ($p = 0.02$, Figure 2D). When comparing preschool wheezers with healthy controls, the relative abundance of *Collinsella* ($p = 0.01$) and *Dorea* ($p = 0.02$) was significantly lower in wheezing children (Figure S3). Finally, we examined whether gut microbial profiles were related to atopic sensitisation, Tregs and *Foxp3* gene expression. Besides a weak, yet statistically significant, positive correlation between *Foxp3* gene expression and bacterial diversity (Shannon index) within the entire study population (Spearman's $\rho = 0.16$; $p = 0.02$), atopic sensitisation, Tregs and *Foxp3* gene expression were neither associated with the overall microbial community structure (Bray-Curtis dissimilarity) nor with the abundance of specific bacterial genera.

To our knowledge, this is the first study to examine the gut microbiota in wheezing preschool children and its association with asthma progression. Our findings suggest that at age 2-4 years, the microbiota perturbations associated with asthma might only be modest. This is in line with the proposed early window-of-opportunity, the first months of life, during which the microbiome is thought to have its strongest impact on immune maturation and tolerance development, and stabilises beyond infancy [1, 9]. However, this early time-window might not be a suitable age to identify biomarkers for asthma prediction as early asthma symptoms may not have occurred yet.

Moreover, ~~some~~ the bacterial genera *Gemmiger* and in particular *Escherichia* were significantly associated with asthma, suggesting that some microbial dysbiosis might still exist at preschool age among wheezing children prone for developing asthma. In a recent paediatric study, *Escherichia* was one of three significantly predominant genera in children with asthma compared to healthy controls [10]. Furthermore, in a recent adult study, *Escherichia* was one of two genera that discriminated asthmatics with fixed airway obstruction from those with no airway obstruction [11]. These results are in line with our findings, potentially indicating that the abundance of *Escherichia* in particular may play a role in the early development of asthma. It has been suggested that an increased abundance of aerotolerant bacteria might be a nonspecific response to inflammatory conditions [12]. Recently, an increase in *Escherichia* appeared to reduce butyrate production, which was associated with asthma [10]. Potentially, *Escherichia* abundance has a wider influence on short-chain fatty acids production, thereby causing an immunological dysbalance leading to a Th2-response.

The strength of our study is that we assessed the gut microbial profiles of a large group of preschool wheezing children, using modern sequencing techniques. Another strength is a reliable asthma diagnosis at age 6 years based on symptoms, medication use, and lung function measurements [8].

The current study has several limitations. First of all, microbiome data are high dimensional, and it cannot be excluded that the observed associations are the result of multiple comparisons. Replication in future studies is therefore needed. Additionally, albeit being the first study to examine the gut microbiota in wheezing preschool children and its association with asthma progression, the sample size limits the power of stratified analyses. The number of sensitised preschool children might for example have been too low to detect significant associations when stratifying for atopic and non-atopic asthma. It is also plausible that microbial perturbations especially impact the risk of asthma in children with certain genetic asthma risk loci, which requires stratified analyses for genotype. Finally, the number of children with eczema was substantial in our population, which might have been accompanied by dietary adaptations. Unfortunately, we had no additional information on the children's diets or restrictions.

In conclusion, gut microbial diversity and overall gut microbial community structure at age 2-4 years was not associated with preschool wheezing or future asthma development at age 6. When compared to microbial perturbations during infancy, microbial perturbations at preschool age appear to be only modestly associated with asthma. On a genus level some bacterial genera were associated with wheezing (*Collinsella* and *Dorea*) or subsequent development of asthma (*Gemmiger* and *Escherichia*), suggesting some microbial dysbiosis in children prone for

developing asthma. The role of these genera in the development of asthma warrants further investigation.

Figures

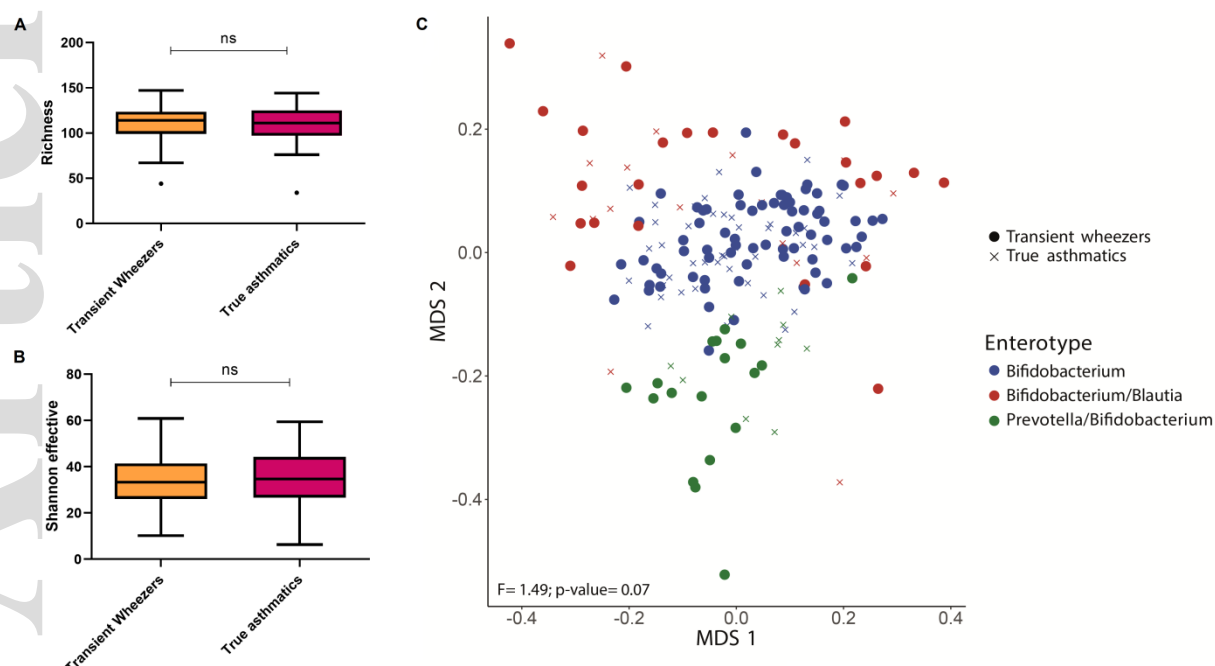


Figure 1. Microbial richness, diversity, and community structure among preschool wheezing children who did (true asthmatics) or did not (transient wheezers) subsequently develop asthma.

Microbial richness (observed species) (**A**) and diversity (Shannon effector index) (**B**) are not significantly different between transient wheezers and true asthmatics (ns: non-significant; Kruskal-Wallis). **C**: Multidimensional scaling (MDS) based on Bray-Curtis dissimilarity indicates three different enterotypes driven by Bifidobacterium, Bifidobacterium/Blautia and Prevotella/Bifidobacterium. Overall microbial community structure is not statistically significantly different between transient wheezers and true asthmatics (permutational analysis of variance (PERMANOVA)).

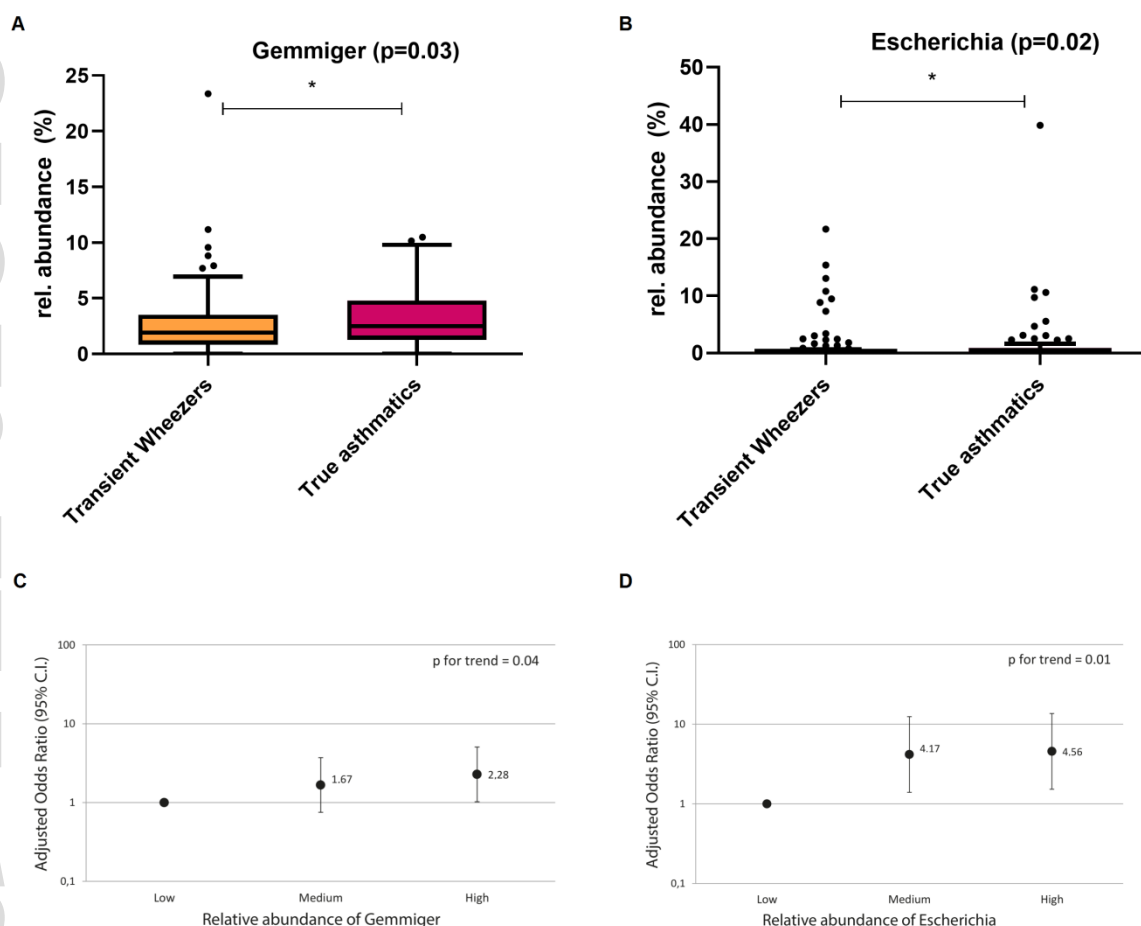


Figure 2. The relative abundance of specific microbial taxa increases the risk of subsequent asthma. The relative abundance of bacterial genera *Gemmiger* (p=0.03) and *Escherichia* (p=0.02) is higher in true asthmatic children compared to transient wheezers (A, B). Multiple logistic regression analyses show that the higher risk to develop asthma among children with a high abundance of *Gemmiger* and *Escherichia* remained statistically significant upon adjustment for sex, breastfeeding, birth season, atopy parents, siblings, parental smoking status, day care attendance (C, D).

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Funding

This study was funded by the Dutch Foundation for Asthma Prevention (project number SAB 2017/006).

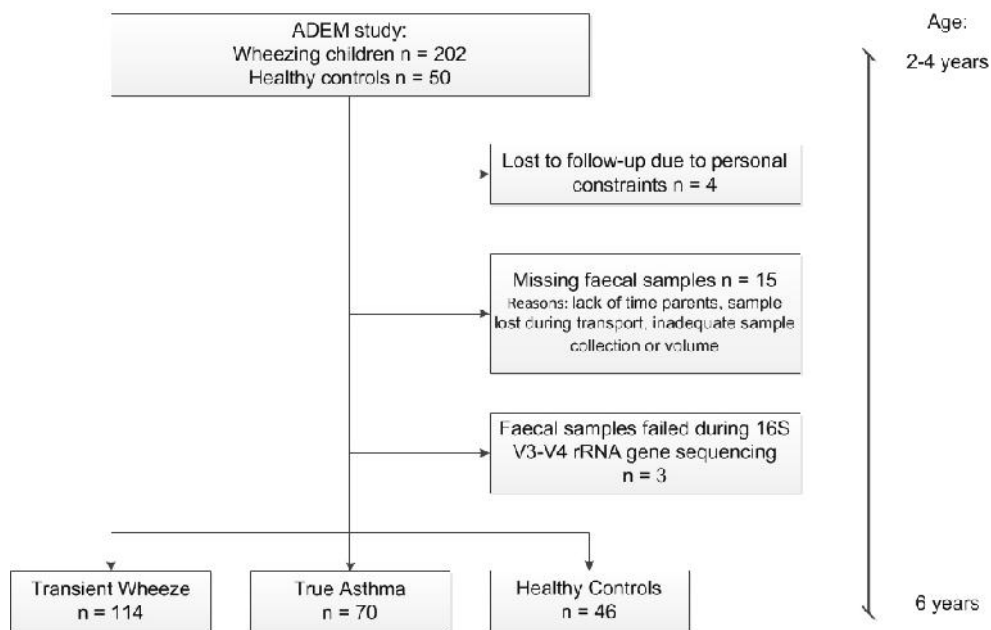
Conflicts of interest

All authors declare that they have no conflicts of interest.

Author Contributions

M.B. and N.v.B. drafted the manuscript, N.v.B., L.B., and J.P. were responsible for gut microbial analysis, M.B., N.v.B., L.B., K.v.d.K., Q.J., E.D., and J.P. contributed to the design of the study, data collection, and interpretation of data. All authors read, revised, and approved the final manuscript.

1 **SUPPLEMENTARY MATERIAL**
2 **TABLES and FIGURES**



3
4 **Figure S1.** Study flow chart.
5

Subject Characteristics	Controls (N=46)	Transient wheeze (N=114)	Asthma (N=70)
Age (years), mean (SEM)	3.5 (0.12)	3.3 (0.07)	3.3 (0.08)
Sex: male / female (n/n)	22/24	60/54	42/28
Atopy*	22	18	33
Allergic rhinitis	2	2	7
Eczema**	24	34	44
Use of inhaled corticosteroids***	0	12	30
Use of short acting β_2 -agonists***	0	28	51
Use of long acting β_2 -agonists**	0	2	6
Day-care attendance	65	75	73
Exposure to furry pets	57	46	46
Passive smoking	35	28	30
Breast feeding	65	61	73
First degree relative with atopic constitution [†]	63	72	74

6 **Table S1: Baseline characteristics of the study population.** Results are displayed as %, unless
7 stated otherwise. Abbreviation SEM: standard error of the mean. *Significantly different between
8 children with transient wheeze and children with asthma ($p < 0.05$); **Significantly different between
9 healthy controls and children with asthma ($p < 0.05$); ***Significantly different between all groups ($p <$
10 0.05); [†]First-degree relative with eczema, asthma or allergic rhinitis.
11

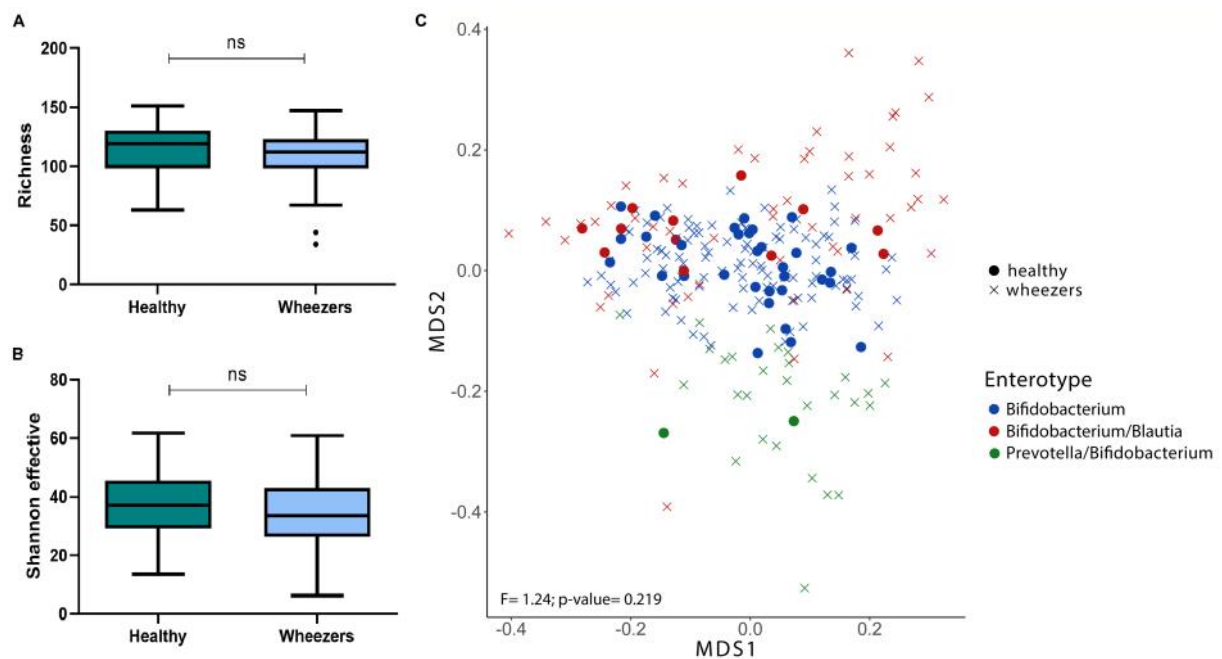


Figure S2. Microbial richness (observed species), microbial diversity (Shannon effector index), and microbial profiles of all children clustered into different enterotypes. Microbial richness (A) and diversity (B) are not significantly different between healthy and wheezing children (ns: non-significant; Kruskal-Wallis). C: Multidimensional scaling (MDS) based on Bray-Curtis dissimilarity indicates three different enterotypes of Bifidobacterium, Bifidobacterium/Blautia and Prevotella/Bifidobacterium in the general microbial community structure. Microbial community structure is not statistically significantly different between healthy and wheezing children (permutational analysis of variance (PERMANOVA)).

Enterotypes	n/N (%) ^s	OR _{adj} [*]	95% CI	p-value
<i>Bifidobacterium</i> ‡	38/105 (36.2)	1.0	-	-
<i>Bifidobacterium-Blautia</i>	22/51 (43.1)	1.454	0.707-2.994	0.31
Prevotella	10/28 (35.7)	0.707	0.276-1.809	0.47

Table S2. Association between enterotype status and childhood asthma.

^sNumber of children with asthma/total number of children in the given enterotype (proportion of children with asthma in enterotype)

‡ Reference category

^{*}Adjusted for sex, breastfeeding, birth season, atopy parents, siblings, parental smoking status, day care attendance.

Enterotypes	n/N (%) [§]	OR _{adj} [*]	95% CI	p-value
<i>Bifidobacterium</i> ‡	105/137 (76.6)	1.0	-	-
<i>Bifidobacterium-Blautia</i>	51/63 (80.1)	1.167	0.537-2.540	0.70
<i>Prevotella</i>	28/30 (93.3)	4.282	0.940-19.51	0.06

Table S3. Association between enterotype status and childhood wheeze.

[§]Number of children with wheeze/total number of children in the given enterotype (proportion of children with wheeze in enterotype)

‡ Reference category

^{*}Logistic regression model Adjusted for sex, breastfeeding, birth season, atopy parents, siblings, parental smoking status, day care attendance.

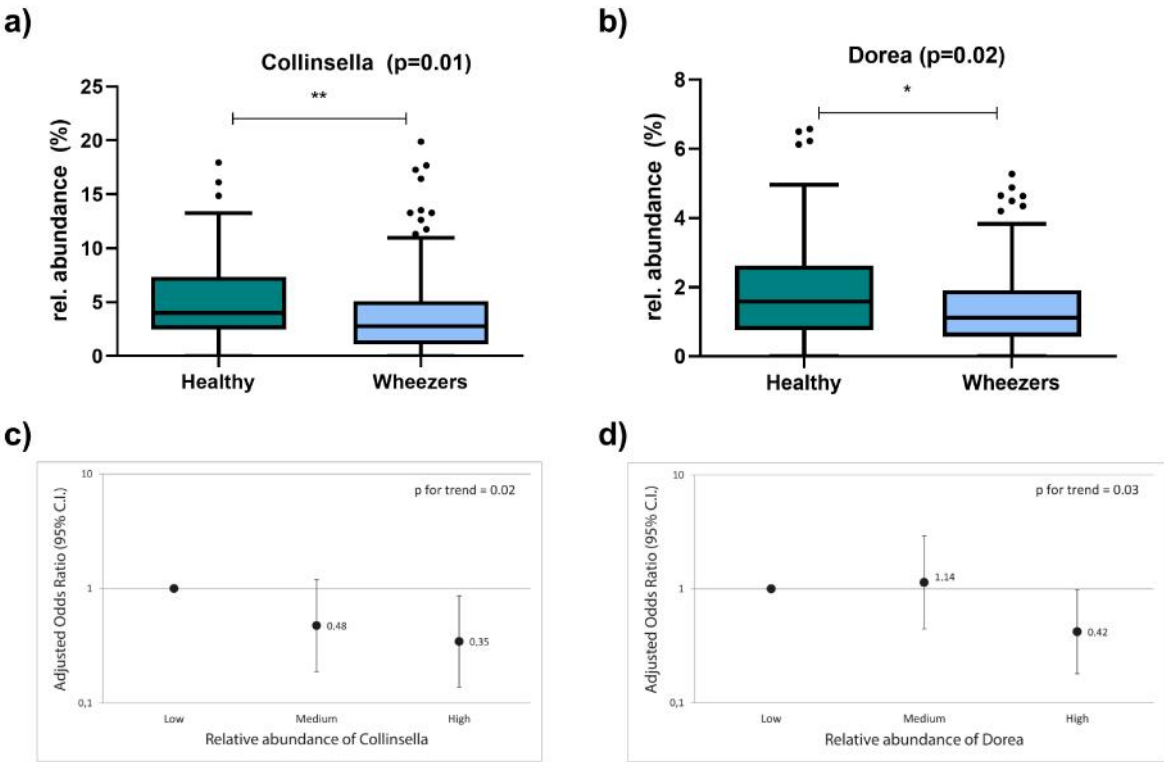


Figure S3. The relative abundance of specific microbial taxa decreases the risk of wheeze. The relative abundance of bacterial genera *Collinsella* (p=0.01) and *Dorea* (p=0.02) is lower in wheezing children compared to healthy controls (**A, B**). The adjusted odds ratio for wheeze according to the relative abundance of the genera as derived from multiple logistic regression analysis show a higher risk to develop wheeze with a low abundance of *Collinsella* and *Dorea* (**C, D**). Adjusted for sex, breastfeeding, birth season, atopy parents, siblings, parental smoking status, day care attendance.

METHODS and RESULTS

DNA isolation and amplicon sequencing of the 16S rRNA V3-V4 gene region

By using a standardised protocol, faeces of all participating children was collected at inclusion [1]. After reception, samples were immediately frozen at -80 °C until further analysis. DNA isolation was performed using a combination of mechanical (repeated bead beating) and chemical lysis followed by column-based purification as described previously [2]. For each DNA isolation batch, additional isolation was performed on PCR-grade water as a negative control. The V3-V4 region of the 16S rRNA gene was PCR amplified from each DNA sample in triplicate. Pooled amplicons from the triplicate reactions were purified using AMPure XP purification (Agencourt, Massachusetts, USA) according to the manufacturer's instructions and subsequently quantified by Quant-iT PicoGreen dsDNA reagent kit (Invitrogen, New York, USA). Amplicons were mixed in equimolar concentrations, to ensure equal representation of each sample, and sequenced on an Illumina MiSeq instrument. Amplicon libraries of the 16S rRNA V3-V4 gene region and subsequent sequencing (Illumina, MiSeq) was performed by Baseclear [Leiden, the Netherlands]. The sequence-data generated in this study have been deposited to the EBI/ENA and Qitta databases under accession No. ERP105097 and 11450 respectively.

For subsequent data analysis, we used a UPARSE based analysis pipeline [3]. Here, demultiplexing was performed by demultiplexor_v3.pl (unpublished Perl script). Pairing, quality filtering and OTU clustering (97% identity) was done by USEARCH 8.0 [4]. Chimera filtering by UCHIME (with RDP set 15 as a reference database) [5]. Taxonomic classification by RDP classifier version 2.11 training set 15 [6]. Sequence alignment by MUSCLE and treeing by Fasttree [7, 8].

Data demultiplexing, length and quality filtering, pairing of reads and clustering of reads into Operational Taxonomic Units (OTUs) at 97% sequence identity was done using the online Integrated Microbial Next Generation Sequencing (IMNGS) platform [9], using default settings except for minimum and maximum length for amplicons which were set at 300 and 600 bp respectively. IMNGS is based upon the UPARSE algorithm [3].

Data normalisation, diversity, taxonomical binning and group comparisons were performed using Rhea package [10]. In order not to discard informative data, normalisation in Rhea is performed by dividing OTU counts per sample for their total count (sample depth) followed by multiplying all of the obtained relative abundance for the lowest sample depth (8,278 reads/sample). Alpha- (observed species, Chao1 and Shannon index) and beta-diversity (generalised Unifrac, Bray-Curtis dissimilarity) estimates were computed in R [11].

Power analysis

We calculated the power based upon the sample size of our study consisting of 70 asthma cases and 114 transient wheezers (controls). There is 80% power to detect significant differences ($\alpha = 0.05$) in the relative abundance of 2 OTUs with 61% and 3 OTUs with 70% using the following settings: MonteCarlo replications = 1,000, Shape π_1 = Stool, #OTUs = 85 [12].

Atopy

Atopy was defined positive when a specific IgE concentration against a mixture of inhalant and food allergens was ≥ 0.35 kU/L (Phadiatop Infant test; Phadia, Uppsala, Sweden).

IgE-mediated asthma was defined as children with a diagnosis of asthma at age 6 years, and specific IgE levels ≥ 0.35 kU/l against one or more aeroallergens as determined by the Phadiatop Infant test.

Regulatory T cells

Tregs ($CD4^+CD25^{\text{high}}CD127^-$) were determined by flow cytometry. As published before, the $CD4^+CD25^{\text{high}}CD127^-$ phenotype was shown to be a valid surrogate for *Foxp3* positive Tregs in our study population [13].

Foxp3 gene expression

Total RNA was extracted from peripheral blood mononuclear cells (PBMC), and expression of *Foxp3* gene was assessed. A detailed study procedure was previously published [14].

Data analysis

IBM SPSS Statistics 20, SAS 7.30 and R statistical software were used for data analysis. To test for statistical significant differences in the overall microbial community structure between study groups, permutational multivariate analysis of variance (PERMANOVA) using distance matrices (vegan::adonis) were performed. Enterotyping, using Dirichlet Multinomial Mixture (DMM) clustering, was performed as described previously [15], in order to cluster faecal samples based upon specific microbial community structures. To examine whether the study group associated with specific enterotypes, multivariable logistic regression analyses were performed while adjusting for potential confounders (sex, breastfeeding, birth season, parental atopy, siblings, parental smoking status, day care). To test whether specific microbial taxa and/or microbial diversity differed between study groups (e.g. healthy controls vs. wheeze or transient wheeze vs. asthma), Kruskal-Wallis tests and Mann-Whitney U tests were used. For those bacteria that appeared to be statistically significantly different between the study groups in univariable analyses, multivariable logistic regression analyses were performed to examine whether these associations remained upon adjustment for potential confounders (see above). To identify possible relations between microbial richness, diversity and genus abundance and continuous outcomes (i.e. *Foxp3* gene-expression and Tregs) spearman correlations were used.

RESULTS

Microbiota

A total of 12,230,374 V3-V4 reads were generated. After trimming, quality filtering, removal of potential chimeric reads, de-multiplexing, and removal of low abundant operational taxonomic units (OTUs), 6,885,888 paired-end sequences belonging to 336 OTUs were retained for downstream analysis. Samples of three participants failed during sequencing and were excluded from subsequent analysis.

For the remaining samples of 230 children (70 asthmatics, 114 transient wheezers, and 46 healthy controls), the number of sequences per sample ranged from 2,362 to 44,357 (median 30,471).

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